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REGULAR ARTICLE

Soil biodegradation of a benzoxazinone analog proposed as a natural products-based herbicide

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Abstract
Aims Benzoxazinones with the 4-hydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one skeleton have been proposed as potentially successful models for the development of novel design leads. D-DIBOA has proven to be the most promising base structure in the search for novel herbicide models based on the benzoxazinone skeleton. The biodegradation dynamics of D-DIBOA in soil are therefore relevant and are the subject of this study.
Methods A previously optimized methodology for the assessment of biodegradation has been applied for the first time to a synthetic benzoxazinone.
Results Biodegradability is a characteristic of natural benzoxazinones and a safety requirement for the development of herbicidal chemicals. The biodegradation phenomenon and its consequences for the development of new herbicide models are discussed. The half-life determined for D-DIBOA was much higher than those previously reported for the natural products DIBOA, DIMBOA and their benzoxazolinone derivatives.
Conclusions This finding, together with its previously described potent phytotoxicity, suggests that D-DIBOA is a useful candidate for novel herbicide model development. The lactam D-HBOA, which is slightly less phytotoxic than its precursor, was discovered to be the first and principal metabolite resulting from D-DIBOA degradation.

Keywords D-DIBOA · Benzoxazinones · Degradation study · Soil · Herbicide models

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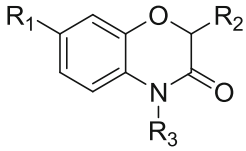
Introduction

Natural products have long been used as pesticides and have widely served as a source of inspiration for many commercial synthetic organic fungicides, herbicides and insecticides that are on the market today (Gerwick and Sparks 2014). One of the important benefits of natural products is that most of these compounds are rapidly degraded in the natural environment, a characteristic that accounts for the perception that most natural products are environmentally benign. However, this is possibly one of the Achilles' heels of natural products. The rate of degradation of natural products may also be too rapid to allow their development as successful herbicides (Dayan et al. 2012).

The possible application of benzoxazinones in the search for new natural herbicide models is a topic of current interest due to the wide variety of bioactivities and the ecological roles exhibited by natural benzoxazinones and some of their related compounds (Tables 1, 2) (Argandoña et al. 1983; Barnes and Putnam 1987; Bravo and Lazo 1996; Perez and Ormeno-Nunez 1991; Schulz et al. 2013).

Table 1 Natural benzoxazinones and synthetic derivatives mentioned in this study



	R_1	R_2	R_3	Acronym
Natural Allelochemicals (A)	H	O-β-D-Glc	OH	DIBOA-Glc
	H	OH	OH	DIBOA
	OCH_3	OH	OH	DIMBOA
Degradation Products (B)	H	OH	H	HBOA
	OCH_3	OH	H	HMBOA
Synthetic analogs (C)	H	H	H	D-HBOA
	OCH_3	H	H	D-HMBOA
	H	H	OH	D-DIBOA
	OCH_3	H	OH	D-DIMBOA
	H	H	OAc	ABOA
	OCH_3	H	OAc	AMBOA

Given the potential for the development of natural herbicide models of some of the synthetic benzoxazinone derivatives (Macias et al. 2005a, b, 2006b), it is necessary to characterize the behavior of such derivatives in a natural ecosystem. Laboratory studies on the phytotoxic effects of some synthetic derivatives of the benzoxazinones DIBOA and DIMBOA yielded promising results and these compounds therefore represent new leads for the development of chemicals that would be useful in weed control as natural templates.

From the point of view of the ecological role and potential applicability of benzoxazinones and related compounds in the development of novel herbicide models, it is interesting to characterize the biodegradability of these chemicals in soil (Cipollini et al. 2012). The stability of benzoxazinones and their derivatives has been extensively investigated, especially for natural benzoxazinones and benzoxazolinones (Fomsgaard et al. 2004; Macías et al. 2007; Oliveros-Bastidas et al. 2009).

As the degradation processes yield a wide variety of chemical structures, each one with different phytotoxic effects, the degradation phenomena observed for plant phytotoxins have interesting ecological implications (Macías et al. 2007). In fact, hydroxamic acids with the benzoxazinone skeleton yield lactams (Table 1), benzoxazolinones, aminophenoxazines, malonamic acids, aminophenols and acetamidophenols (Table 2) in different environments and under different conditions (Oliveros-Bastidas 2006). The presence and relative proportions of these chemicals in soil affects the chemoecological interactions between donor and target species, as demonstrated by recent findings (Macias et al. 2014).

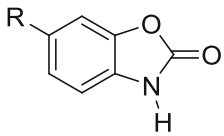
The phytotoxicity of benzoxazinones and their degradation products on Standard Target Species (Macias et al. 2005b) and common weeds (Macias et al. 2005a, 2006b) has been evaluated. These reports offer a systematic comparison, under the same conditions, of benzoxazinones (hydroxamic acids and lactams), benzoxazolinones, aminophenoxazines, malonamic acids and aminophenols with a commercial herbicide (terbutryn 59.4 %, triasulfuron 0.6 %). The relative phytotoxicity was presented for each species and the synthetic benzoxazinones 4-hydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (D-DIBOA) and 4-acetoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (ABOA) were found to be the most phytotoxic (Fig. 1).

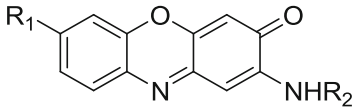
The lack of a hydroxyl group at position C-2 for these synthetic benzoxazinones, which was found to be closely associated with an increase in phytotoxicity, was proposed to enhance the stability of the benzoxazinone skeleton. The degradation process from benzoxazinones to benzoxazolinones is related to hemiketal cleavage at C-2 in aqueous solution, in which the rate of this process is closely related to pH (Macias et al. 2005a, b, 2006b).

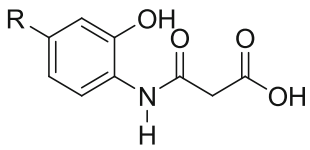
The presence of a hydroxyl group at C-2 is the key for all DIBOA and DIMBOA degradation mechanisms. Thus, 2-deoxybenzoxazinones such as D-DIBOA could show very different degradation behavior and different metabolites could therefore appear in the soil after their application as pest management tools, which in turn could have potential consequences on their efficacy and environmental side-effects. In the work described here, degradation experiments were carried out in wheat crop soil and D-DIBOA was evaluated using previously reported methodologies for analysis (Eljarrat et al. 2004) and degradation kinetics (Macias et al. 2004, 2005c).

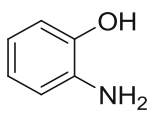
The choice of D-DIBOA as a model for phytotoxic benzoxazinones resulted in significant improvements in phytotoxicity and selectivity. The systematic esterification of D-DIBOA at the N-4 or C-2 positions resulted in a new generation of esters with increased lipophilicity. Stronger phytotoxic effects and higher selectivity were observed for these compounds in comparison to natural benzoxazinones (Macías et al. 2008; Macias et al.

Table 2 Benzoxazinone degradation products discussed in this study

Benzoxazolinones	R	Acronym
	H	BOA
	OCH_3	MBOA

Aminophenoxazines	R_1	R_2	Acronym
	H		APO
	OCH_3	H	AMPO
	H	OAc	AAPO
	OCH_3	OAc	AAMPO
	OH	H	AHPO

Malonamic acids	R	Acronym
	H	HPMA
	OCH_3	HMPMA

Miscellaneous	Acronym
	APH

2006d). Among them, 4-propanoyloxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (Pr-D-DIBOA) and 2-ethyl-(2*H*)-1,4-benzoxazin-3(4*H*)-one (2-Et-D-DIBOA) were proposed as the optimal models for phytotoxic action and selectivity. These aspects were assessed by statistically comparing their effects on *Avena fatua* and *Echinocloa crus-galli* with those recorded on wheat and rice (Fig. 2).

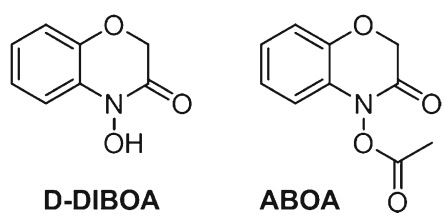


Fig. 1 Structures of 4-hydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (D-DIBOA) and 4-acetoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (ABOA)

Furthermore, improvement of the benzoxazinone skeleton by modification of the steric and electronic features was carried out (Macias et al. 2006a, 2009). Halogenated models 6-chloro-(2*H*)-1,4-benzoxazin-3(4*H*)-one (6Cl-D-DIBOA), 6-fluoro-(2*H*)-1,4-benzoxazin-3(4*H*)-one (6 F-D-DIBOA) and 7-fluoro-(2*H*)-1,4-benzoxazin-3(4*H*)-one (7 F-D-DIBOA) were chosen for their phytotoxic effects, with the latter compound having a remarkable *A. fatua*-wheat selectivity, by comparison of their IC50 values on each species bioassay (Macias et al. 2006a). Optimal ranges for molecular volume, dipole moment and polarizability of benzoxazinones to maximize the phytotoxic activity were characterized (Fig. 2). Finally, considering the promising results obtained for some of the derivatives, the synthesis and bioactivity evaluation of models including more than one kind of structural modification

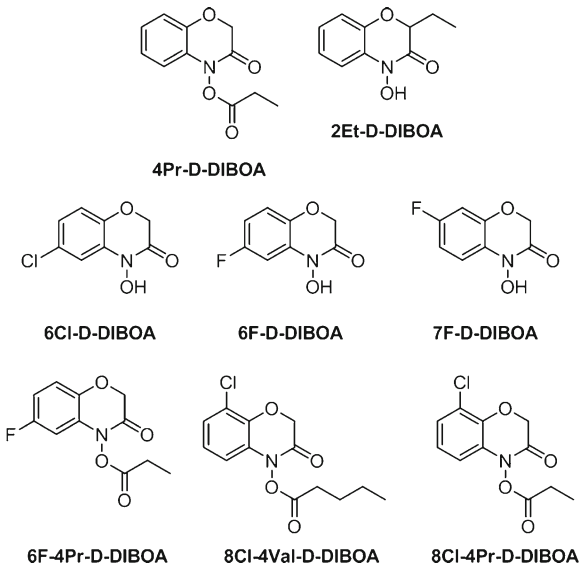


Fig. 2 Structures of the more active synthetic derivatives of D-DIBOA

were planned. In this respect, modification of the aromatic ring was combined with the modulation of lipophilicity in positions N-4 or C-2. 6-Fluoro-4-propionyloxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (6 F-4Pr-D-DIBOA), 8-chloro-4-valeryloxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (8Cl-4Val-D-DIBOA) and 8-chloro-4-propionyloxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (8Cl-4Pr-D-DIBOA) were the derivatives that showed the best bioactivity and selectivity profiles in the systems *A. fatua*-wheat and *E. crus-galli*-rice (Fig. 2) (Macias et al. 2010; Macías et al. 2010).

These findings suggest that D-DIBOA and its derivatives have great potential as agrochemicals. It is therefore of considerable interest to assess the stability of these compounds in soil cultures in the context of their possible use as models for the development of new herbicides. The biodegradation phenomena for D-DIBOA and their consequences are discussed in this work.

Material and methods

Soil sampling and experiment design

Soil samples were collected randomly from a cultivated crop field located at La Barca de la Florida, Cádiz, Spain (36° 38′ 28.18″ N, 5° 57′ 43.14″ W). The samples were taken from the vicinity of the radicular system, with a maximum horizontal distance of 15 cm from the shoot, and a maximum depth from the ground surface of 10 cm. There were no other plant species present in the soil at this time. The wheat was in its N° 59 ('end of heading') growth stage according to the BBCH growth stage classification, approximately 250 days after planting (Meier 1997). The collected soil samples were stored in plastic bags at −15 °C prior to use. Before degradation experiments were performed, wheat soil was maintained at ambient temperature and dried for 2 days. All remaining vegetation and stones were removed manually and soil was sieved (<1 mm) and placed into 50 mL sterile glass vials (2 g each). One mL of a 2 mg/mL D-DIBOA sterile solution in deionized water (filter sterilization, <2 μm) was placed into each vial. Two control sets were used: one set without soil to assess non-biocatalyzed degradation ('witness solution') and one with sterile soil (previously autoclaved) to record the influence of inorganic soil composition on degradation rates. All samples were placed in a growth chamber (25 °C, 16 h light/8 h darkness) for the duration of the experiment. Sterile and non-sterile samples, together with an aliquot of 'witness solution', were collected at 0, 2, 4, 6, 8, 10, 12 and 29 days (three replicates for each sampling time). After collection, 1 mL of MeOH was added to each sample to avoid subsequent microbial degradation. The samples were stored at −15 °C prior to analysis.

Analytical methodology and standardization

For HPLC analysis, soil solutions were centrifuged in a Selecta Microfiger BL 71379 apparatus at 13,000 rpm for 10 min and then filtered (<44 μm). The resulting solid residue was extracted with methanol (10 mL) in an ultrasonic bath (15 min, 5 °C). The extract was centrifuged again for 10 min and the process was repeated three more times. This process was repeated using ethyl acetate (10 mL) as solvent. The soil aqueous solution, the methanol, and the ethyl acetate extracts were distilled under reduced pressure. The solid residues were dissolved in MeOH (2 mL) with 1 % acetic acid and then filtered (<0.2 μm) prior to HPLC injection. All samples were analyzed on a Merck HITACHI HPLC system equipped with a LaChrom L-7100 quaternary gradient pump, an L-7455 LaChrom diode array detector and an L-7200 LaChrom autoinjector. Data were collected and processed using an HPLC data system (Merck HITACHI D7000). Instrument conditions for analysis were: Lichrospher 100 RP-18 (250×4.0 mm,

Table 3 Calibration curves for the DIBOA degradation series

Abs=*A* [chemical]+*B*					
Chemical	λ_{max}	A	B	R^2	RT (min)
D-DIBOA	280	$2{\cdot}10^7$	10^6	0.9854	14.75
D-HBOA	280	$2{\cdot}10^7$	10^6	0.9862	15.55
DIBOA	253	$2{\cdot}10^7$	175074	0.9990	9.95
BOA	264	10^7	$2{\cdot}10^6$	0.9681	14.67
APH	269	10^7	60279	0.9995	3.44
HPAA	278	10^7	642363	0.9912	10.48
APO	260	$3{\cdot}10^7$	241476	0.9990	22.53

5 μm) reversed-phase column at 25 °C. Mobile phases were water:1 % AcOH (A) and methanol:1 % AcOH (B) at a flow rate of 1 mL min^{-1}. The injection volume was 50 μL. The following gradient was used for separation: at 0 min, 30 % B; 2 min, 30 % B; 19 min, 60 % B; 21 min, 100 % B. Under these conditions, the D-DIBOA retention time was 14.75 min. A calibration curve was created for D-DIBOA at λ=280 nm (1, 0.5, 0.25, and 0.125 mg/mL). The equation for the curve was $Abs=2{\cdot}10^7[\text{D-DIBOA}]+1{\cdot}10^6$ (R^2=0.9854).

In order to identify degradation products from D-DIBOA, several metabolites belonging to the natural DIBOA degradation series, together with lactam D-

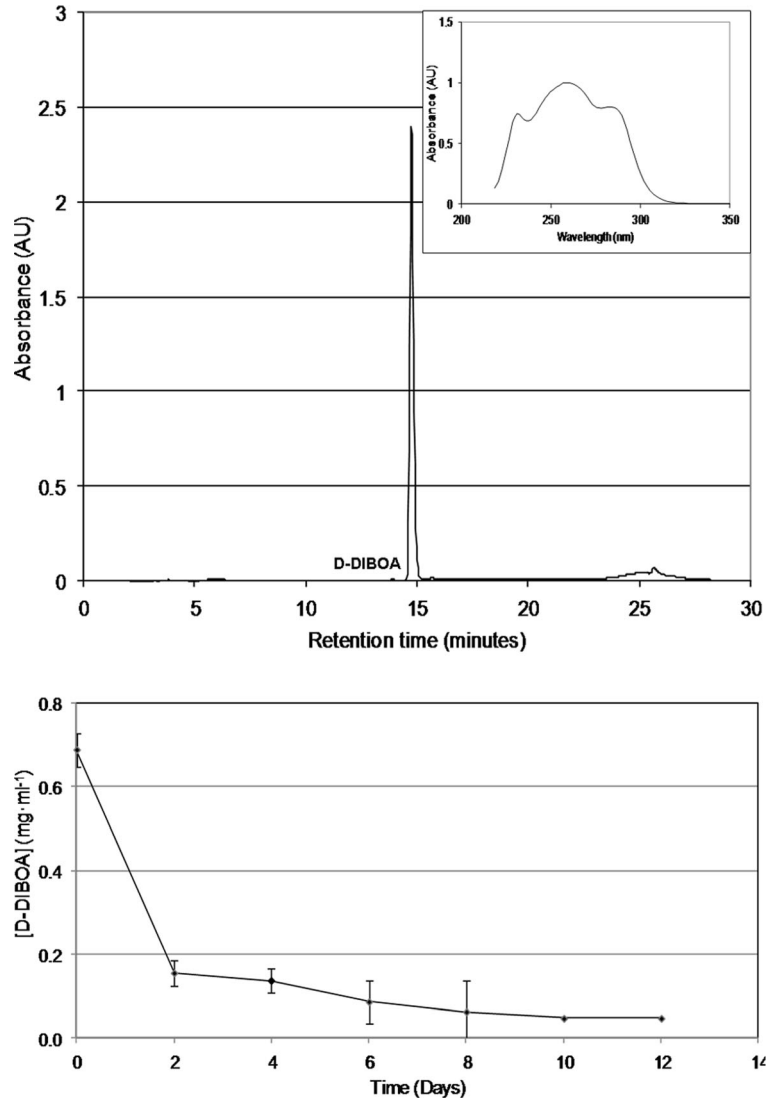


Fig. 3 *Top*: D-DIBOA chromatogram with UV detection at 280 nm, and UV–vis spectrum. *Bottom*: Variation of D-DIBOA concentration with time

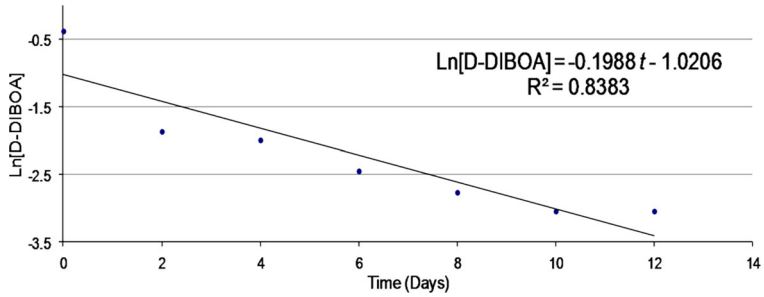


Fig. 4 Average regression curve for D-DIBOA concentrations

HBOA, were injected under the same conditions and the corresponding calibration curves were built (Table 3).

2-Aminophenol (APH) and 2-acetamidophenol (HPAA) were purchased from Sigma-Aldrich Co. and were used in assays. BOA was obtained from Fluka Chemika and was used as received. D-DIBOA, D-HBOA and APO were obtained by the synthetic methods previously described by (Macias et al. 2006c).

D-DIBOA was degraded in wheat crop soil and its half-life in this environment was determined on the basis of the data points from the three determinations, treated independently. The structure of the main degradation product (D-HBOA) is proposed on the basis of its spectrophotometrical properties and by comparison with the synthetic standard.

Results

Degradation of D-DIBOA with time

The evolution of D-DIBOA concentration with time is shown in Fig. 3 together with a standard chromatogram and the UV–vis spectrum. Degradation was not observed in sterile control samples or in the witness solution.

The concentration data show a very steep decay in the D-DIBOA concentration from t = 0 to t = 2 days. The concentration continued to decay up to the 12th day. After this period, the system reached equilibrium and the concentration remained constant. The linear decay range to fit the data to 1st order kinetics was established from t = 2 to t = 12 days. The calculations provided a half-life $t_{1/2}$=3.6±0.2 days. The average regression curve is shown in Fig. 4. The average recovery of D-DIBOA at t = 0 was 0.69±0.04 mg·mL^{-1}.

D-DIBOA degradation metabolites

Only one of the compounds chosen as potential degradation chemicals (see Table 1) was detected in the D-DIBOA degradation experiment, namely lactam D-HBOA, which was detected after 48 h (Fig. 5).

The evolution of D-HBOA concentration with time is shown in Fig. 6. A slight increase in concentration was observed up to day 19. After this time, the concentration

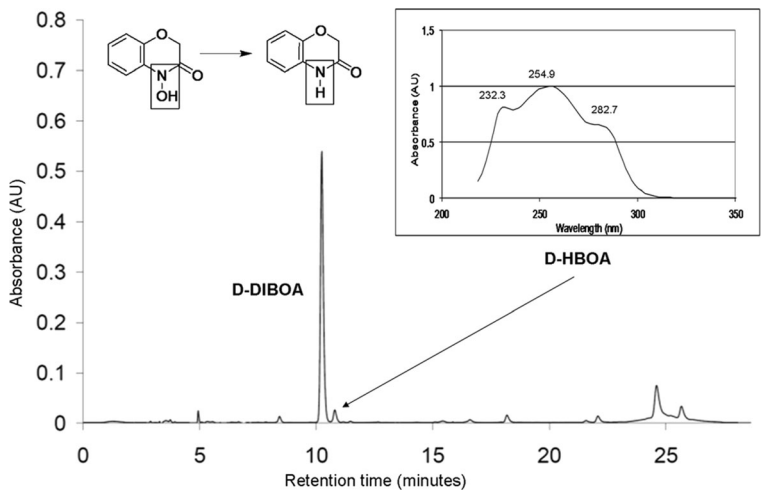


Fig. 5 D-DIBOA and D-HBOA signals, D-HBOA UV–vis spectrum

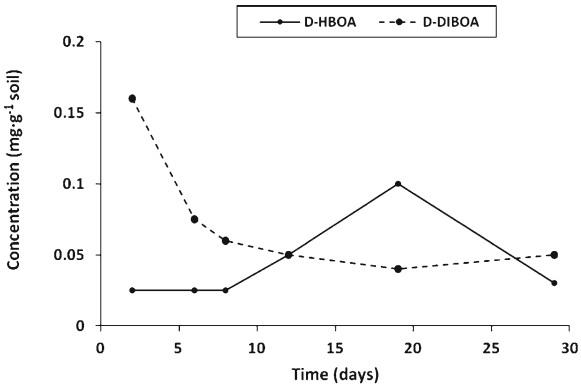


Fig. 6 Comparison of D-DIBOA and D-HBOA concentrations in soil. Values shown for sampling times of 2, 6, 8, 12, 19, and 30 days

of D-HBOA decreased rapidly. Other unknown metabolites appeared after day 20 (none of which belonged to the compounds used as standards) and it is therefore evident that D-HBOA is metabolized into chemicals other than those belonging to the DIBOA degradation series characterized by us (Macias et al. 2005c). D-HBOA is also likely to be degraded over time, as shown in Fig. 6.

The first degradation product from D-DIBOA in wheat crop soil begins to degrade only a short time after it first appears. D-HBOA is less active than D-DIBOA (Macias et al. 2005b). Thus, this degradation process leads to a loss of phytotoxicity, at least during the initial stages.

Discussion

The biodegradability of D-DIBOA was studied by a previously reported methodology (Macias et al. 2004, 2005a). These experiments provided evidence that the analytical methodology employed for DIBOA and DIMBOA degradation studies is valid to determine the stability of D-DIBOA. A half-life of 3.6±0.2 days was obtained and this is much higher than that previously determined at the same dosage on soil for DIBOA (26 h) (Macias et al. 2005c), DIMBOA (32 min) (Macias et al. 2004) and their benzoxazolinone derivatives (Oliveros-Bastidas et al. 2012). This result confirms the starting hypothesis that greater stability is potentially associated with a higher stability for this 2-deoxybenzoxazinone in typical field soil. Changes in the aromatic substitution pattern modify the stability of benzoxazinones and their derivatives markedly (Macias et al. 2004, 2005c) and one would expect the half-life for D-DIBOA to be longer than that found for DIBOA and the chemicals belonging to its degradation series. Both DIBOA and its glucoside, DIBOA-Glc, had much lower degradation times at the same dose. The values were in the range 0.025–0.030 h. The half-life of D-DIBOA is much higher than that recorded for BOA (0.26–0.31 days).

In addition, the first chemical of the D-DIBOA degradation series was determined to be the lactam D-HBOA, which is slightly less phytotoxic than its precursor. This chemical also degrades and the chemicals resulting from this process are different to those previously observed in DIBOA or DIMBOA degradation studies.

Despite the fact that D-DIBOA lacks a hydroxyl group at C-2, a biodegradation process still takes place and this is promising from the point of view of its potential application as biorational pest management tool as it indicates adequate environmental compatibility.

Our findings suggest that synthetic hydroxamic acids and their derivatives may prove to be useful as novel bioherbicidal molecules. Several of these compounds, including D DIBOA, offer some residual soil activity, which may enable their eventual use as preemergent herbicides after further study.

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Conflict of interest The authors declare no conflict of interest associated with this manuscript.

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